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Strains nodulating *Lupinus albus* on different continents belong to several new chromosomal and symbiotic lineages within *Bradyrhizobium*

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Keywords: *Bradyrhizobium*/*Lupinus*/phylogeny

Abstract

In this work we analysed different chromosomal and symbiotic markers in rhizobial strains nodulating *Lupinus albus* (white lupin) in several continents. Collectively the analysis of their *rrs* and *atpD* genes, and 16S-23S intergenic spacers (ITS), showed that they belong to at least four chromosomal lineages within the genus *Bradyrhizobium*. Most isolates from the Canary Islands (near to the African continent) grouped with some strains isolated on mainland Spain and were identified as *Bradyrhizobium canariense.* These strains are divided into two ITS subgroups coincident with those previously described from isolates nodulating *Ornithopus* . The remaining strains isolated on mainland Spain grouped with most isolates from Chile (American continent) forming a new lineage related to *Bradyrhizobium japonicum*. The strains BLUT2 and ISLU207 isolated from the Canary Islands and Chile, respectively, formed two new lineages phylogenetically close to different species of *Bradyrhizobium* depending on the marker analyzed. The analysis of the *nodC* gene showed that all strains nodulating *L. albus* belong to the biovar genistearum; nevertheless they form four different *nodC* lineages of which lineage C is at present exclusively formed by *L. albus* endosymbionts isolated from different continents.

Introduction

Lupinus albus (white lupin) is a legume which has been cultivated in Europe for the last 2000 years, used in human and animal feeding, as green manure in agriculture (Rosolem *et al.*, 2002; Jensen *et al.*, 2004) and in soil stabilization (Clapham, 1997). This species is currently considered a good alternative as an animal foodstuff due to the high quality of its proteins (Erbas *et al.*, 2005). Therefore there is increasing interest in this plant to be used in sustainable agriculture due to its high potential to provide protein without nitrogen fertilization (Robinson *et al.*, 2000; Dijkstra *et al.*, 2003), estimated at 150-200 Kg of nitrogen per ha in symbiosis with *Bradyrhizobium* (Robinson *et al.*, 2000). Despite the interest of this symbiosis there are few studies about the identity of strains nodulating *L. albus* in different continents whereas several studies have been carried out with other *Lupinus* species (Barrera *et al.*, 1997; Stepkowski *et al.*, 2005; Andam and Parker, 2007; Stepkowski *et al.*, 2007). Although *L. albus* may be also nodulated by strains of *Ochrobactrum* this symbiosis was not very effective (Trujillo *et al.*, 2005). The strains isolated to date from effective nodules of *L. albus* in different countries belong to the genus *Bradyrhizobium* (Barrera *et al.*, 1997; Jarabo-Lorenzo *et al.*, 2003; Stepkowski *et al.*, 2007; Rivas *et al.*, 2009). However these strains have not been extensively analysed and only for a few strains have the same genes been studied. For example, the ARDRA profiles of Canary Island isolates were previously analysed but not their *rrs* gene sequences. Of the Chilean strains, this sequence is only known in the strains ISLU227 and ISLU207 (Jarabo-Lorenzo *et al.*, 2003). Several housekeeping genes have been analysed for the strains isolated in mainland Spain (Stepkowski *et al.*, 2007; Rivas *et al.*, 2009) but the *rrs* gene and the 16S-23S intergenic spacer (ITS) have not been previously analysed. Finally, the *nodC* gene has been only analysed in the strain ISLU207 (Jarabo-Lorenzo *et al.*, 2003).

Therefore the aim of this study was to analyse the phylogenetic relationships of *L. albus* strains isolated on three different continents using three chromosomal markers with different rates of evolutionary divergence (the *rrs* and *atpD* genes and the ITS spacer) and a symbiotic marker, the *nodC* gene, related with the host range of legumes (Roche *et al.*, 1996; Perret *et al.*, 2000, Rivas *et al.*, 2006; Iglesias *et al.*, 2008; Laranjo *et al.*, 2009). The results showed that the *L. albus* endosymbionts belong to several chromosomal lineages within the genus *Bradyrhizobium* that could represent new species of this genus. A group of these endosymbionts constitute a *nodC* lineage that could represent an allelic group present up to date only in *L. albus* bradyrhizobia.

Materials and Methods

Strains and nodulation experiments

The reference and the *L. albus* strains analysed in this study are listed in Table 1. These strains were isolated from *L. albus* nodules in previous studies according to the method of Vincent (1970). For nodulation experiments *L. albus* plants were inoculated with representative isolates, under growth chamber conditions, in modified Leonard jars using vermiculite as substrate and nitrogen-free nutrient solutions. Non-inoculated nitrogen-free and nitrogen-supplemented plants were used as controls. Five replicates were set per treatment and plants were harvested 6 weeks after planting. Shoot dry weight and number of nodules were the parameters measured. Symbiotic efficiency was determined as described by Somasegaran and Hoben (1994). Data were analyzed by one-way analysis of variance, and mean values compared by Fisher's Protected LSD test (Least Significant Differences) ($P \leq 0.05$).

RAPD fingerprinting

RAPD patterns were obtained using the primer M13 (5'- GAGGGTGGCGGTTCT –3') according to Rivas *et al.* (2006) in the following PCR conditions: preheating at 95 ^{o}C for 9 min; 35 cycles of denaturing at 95 ^{o}C for 1 min; annealing at 45 ^{o}C for 1 min and extension at 75 ^{o}C for 2 min, and a final extension at 72 ^{o}C for 7 min. The PCR products were electrophoresed on 1.5% agarose gel in TBE buffer (100 mM Tris, 83 mM boric acid, 1 mM EDTA, pH 8.5) at 6 V/cm, stained in a solution containing 0.5 µg/ml ethidium bromide, and photographed under UV light. Standard VI (Roche, USA) was used as molecular weight marker. An 8 µl aliquot of loading solution (40% sucrose and 0.25% bromophenol blue) was added to each sample. The bands present in each profile were coded for input into a database including all the strains studied and Jaccard's similarity coefficient was calculated to construct the distance matrix. A dendrogram was constructed from the distance matrix using the unweighted pair group with arithmetic mean (UPGMA) using the GelCompar II program from Bionumerics platform.

Analysis of *rrs, atpD* and *nodC* genes and 16S-23S intergenic spacer (ITS)

The *rrs* was amplified and sequenced according to Rivas et al. (2007a), the *atpD* gene according to Gaunt et al. (2001) and the ITS as described by Willems et al. (2003). A partial sequence of the *nodC* gene was obtained by using the primers designed in this study NodCBradyF (5′-CGCAAGGCGCAG(AT)TCGC-3′) and NodCBradyR (5′-GG(GT)GTG(AGC)AGCG(AC)GAAGCCG-3′) in the following PCR conditions: pre-heating at 95°C for 9 min; 35 cycles of denaturing at 95°C for 1 min; annealing at 45°C for 1:30 min and extension at 72°C for 1 min, and a final extension at 72°C for 7 min. The sequences were obtained in an ABI377 sequencer (Applied Biosystems Inc.) using a BigDye terminator v3.0 cycle sequencing kit as supplied by the manufacturer. The sequences obtained were compared with those from GenBank using the BLASTN program (Altschul et al. 1990). Sequences were aligned using the Clustal W software (Thompson et al. 1987). The distances were calculated according to Kimura′s two-parameter method (Kimura 1980). Phylogenetic trees were inferred using the neighbour-joining method (Saitou and Nei 1987). Bootstrap analysis was based on 1000 resamplings. The MEGA 4 package (Tamura et al. 2007) was used for all analyses. Genbank Accession numbers for the sequences determined in this study are provided in Table 2.

Results and Discussion

RAPD fingerprinting

RAPD fingerprinting is a useful tool for genetic diversity analysis of rhizobia allowing the selection of strains for gene sequencing (Valverde *et al.*, 2006, Iglesias *et al.*, 2007; Santillana *et al.*, 2008; Álvarez-Martínez *et al.*, 2009; Ramírez-Bahena *et al.*, 2009). The results of this analysis in the strains isolated from *L. albus* nodules are shown in Figure 1 and Table 1. They presented 19 different patterns (Fig. 1, lanes 6 to 24) that were also different to those of the reference strains (Fig. 1, lanes 1 to 5). The results of the mathematical analysis showed low similarity coefficients among most of *L. albus* strains and with respect to the reference strains. These results imply a high genetic diversity of *Lupinus* strains and confirmed the usefulness of RAPD patterns to analyze the genetic diversity of rhizobial populations. Considering the low similarity values found after the mathematical analysis (lower than 90%) we sequenced the *rrs* gene and the ITS fragment in all *L. albus* strains.

Analysis of *rrs* gene

Considering that the current phylogenetic classification of rhizobia is predominantly based on *rrs* gene sequences (Kuykendall, 2005) the classification of nodule isolates should be performed on the basis of the results of the analysis of this gene. According to the *rrs* gene analyses previously published the strain BLUT1 was identified as *Bradyrhizobium canariense* (Stepkwoski *et al.*, 2007) and the strain ISLU207 was classified in the phylogenetic group of *Bradyrhizobium japonicum* and *Bradyrhizobium* genospecies alpha (Jarabo-Lorenzo *et al.*, 2003). These results are confirmed in the case of strains BLUT1 and ISLU207, whereas strain ISLU227 belongs to an independent lineage formed by several strains isolated in different continents (Figure 2). To this lineage (with 100% identity) belong the strains ISLU227, ISLU213 and ISLU220 isolated from different geographical locations in Chile (South America), the strain FN13 isolated from *Lupinus montanus* in Mexico (North America) and the strains isolated in León (mainland Spain, Europe). In spite of the proximity of León and Salamanca (mainland Spain, Europe), the strains isolated in Salamanca belong to a different lineage that contains most of the isolates from *L. albus* in the Canary Islands and coincides with the species *B. canariense* (identities higher than 99.8%). Two strains isolated on the Canary Islands, BLUT2 and BLUT3, clustered with the genospecies alpha of *Bradyrhizobium* represented by the strain BC-C1 isolated in the same location but from different hosts (identities higher than 99.6%). Finally, the strain ISLU203 isolated from *L. albus* in Chile belongs to the same lineage as the strain BGA-1 isolated from *Teline stenopetala* in Canary Islands (100% identity).

From the *rrs* analysis it was concluded that the strains isolated from *L. albus* in different continents belong to several different lineages that in some cases presented high identity to already known species of genus *Bradyrhizobium*. Nevertheless, the resolution of *rrs* analysis is too low for *Bradyrhizobium* species assignment and other more variable molecules must be studied (Willems *et al.*, 2003; Rivas *et al.*, 2004).

Analysis of 16S-23S intergenic spacer (ITS)

The ITS sequence analysis has been reported as a better tool than 16S rDNA analysis for species delineation within the genus *Bradyrhizobium,* in which ITS sequence similarities higher than 95.5% indicate a genospecies level relatedness (Willems *et al.*, 2003, Rivas *et al.*, 2004). The results of the phylogenetic analysis of ITS sequences (Figure 3) confirmed that the strains isolated from Salamanca (mainland Spain) and most of the strains isolated from the Canary Islands belong to the species *B. canariense*. These strains were divided into two close

ITS groups with identities higher than 98% (gaps not considered) together with several strains isolated in different geographical regions from different *Genisteae* legumes and *Ornithopus*. These two ITS groups coincide with those designed as ITS-I and ITS-II by Safronova *et al.*, (2007) within *B. canariense,* which can be differentiated by the presence of an insert in the strains from ITS-I group. Nevertheless, we found that the insert is smaller (15 nucleotides) than that reported by Safronova *et al.*, (2007) comprising only the sequence "TAGAGACTTAGGTTT" (located from 731 to 745 in the ITS of the strain Oc9 isolated from *Ornithopus* sp.). All Canary Island isolates belong to the ITS-I group, whereas the Salamanca isolates were distributed in the ITS groups I and II.

The strains isolated in León (mainland Spain, Europe), most strains isolated in Chile (South America) and the strain BLup-MR1 isolated from *Lupinus polyphylus* in Germany (Europe) have identical ITS sequences and grouped in a cluster (III) phylogenetically divergent from that formed by the strains identified as *B. canariense* (identities lower than 98%). They presented high identity values (higher than 99%) with respect to *B. japonicum* bv genistearum strain BGA-1, which presented near 100% identity with the strain ISLU256 isolated from *Ornithopus* in mainland Spain (Jarabo-Lorenzo *et al.*, 2003) and strain FN13 isolated from *L. montanus* in Mexico (North America) (Barrera *et al.*, 1997). This high identity value showed that the strains from these two groups probably belong to the same species but this species could not be *B. japonicum*. The low identity (about 95%) found between the strains of this group, that includes *B. japonicum* bv genistearum BGA-1, and *B. japonicum* bv glycinearum LMG 6138^T suggested they could belong to different species and emphasises the need for a revision of the taxonomic status of the current species *B. japonicum*.

The high similarity (99%) found between the strain ISLU207 isolated in Chile (South America) and the strain BC-C1 (group IV) suggested that both strains belong to the genospecies alpha of genus *Bradyrhizobium*.

Finally, and in spite of their high *rrs* gene identity, the strains BLUT2 and BLUT3 isolated in Canary Islands have 96% identity in their ITS sequences, the limit of ITS similarity for species differentiation in genus *Bradyrhizobium* (Willems *et al.*, 2001). However more strains of these groups are necessary to define new species according to the recommendations of the Subcommittee on the taxonomy of *Agrobacterium* and *Rhizobium* (Lindström and Young, 2009).

Analysis of *atpD* gene

In recent years, analyses of housekeeping genes have been performed in *Bradyrhizobium* showing their usefulness in taxonomic and phylogenetic studies (Vinuesa *et al.*, 2005a; Stepkowski *et al.*, 2007; Ramírez-Bahena *et al.*, 2009; Rivas *et al.*, 2009). From these genes, the *atpD* gene has been previously analysed in strains from *Lupinus* isolated in different geographical locations (Stepkowski *et al.*, 2007; Rivas *et al.*, 2009). In these previous studies, some strains isolated from *L. albus* in Spain and the Canary Islands were already included, with the conclusion that they belong to different phylogenetic groups (Stepkowski *et al.*, 2007; Rivas *et al.*, 2009). In this work we have sequenced the *atpD* genes of strains that had not been previously analysed. As the strains presenting identical ITS sequences also have identical *atpD* genes, only a representative strain from each ITS group and geographical location has been included in the phylogenetic analysis (figure 4). Nevertheless, in some cases the phylogenies based on *atpD* genes and ITS fragments were not completely congruent. For example the strain BLUT3 has a distant ITS sequence but close *atpD* gene with respect to the strain BLUT1. Also, the strain BRE-1 from the genospecies beta of *Bradyrhizobium* has a distant ITS sequence but a close *atpD* gene with respect to the strain BGA-1. Finally, the strain ISLU207 has a close ITS sequence but a divergent *atpD* gene with respect to the strain BC-C1. The significance of these findings should be further studied since strains isolated from the Canary Islands are involved in all these exceptions. The special characteristics of rhizobia present in these Islands have been recently reported for strains nodulating *Phaseolus* having common characteristics with strains isolated in North Africa nodulating this legume and in mainland Spain nodulating *Medicago* (Zurdo-Piñeiro *et al.*, 2009).

The representative strains of ITS groups I and II, isolated in Salamanca and Canary Islands, belong to five different subgroups with *atpD* gene identities higher than 97% among them and with respect to the strain *B. canariense* bv genistearum BTA-1^T. These high identity values are in agreement with those obtained after the ITS analysis and confirmed the identification of strains from ITS groups I and II as *B. canariense.* Several strains isolated from other *Lupinus* species in European countries (Poland and Iceland) and several strains isolated from diverse Genisteae legumes in Africa (Morocco) and nearby geographical zones such as Canary Islands also cluster in this group (Vinuesa *et al.*, 2005; Stepkowsky *et al.*, 2007).

The representative strains from ITS group III, isolated in León and Chile, form a cluster phylogenetically divergent from *B. canariense* that also includes strains isolated from other *Lupinus* species mostly in American countries. These strains were close to the strain *B. japonicum* bv genistearum BGA-1 (99.8% identity), that itself presented about 97% identity with respect to the type strain of *B. japonicum* bv glycinearum LMG 6138^{T}. This identity value is at the limit for species differentiation in the genus *Bradyrhizobium* (Ramírez-Bahena *et al.*, 2009) and thus confirms that the taxonomic status of the strains currently included in *B. japonicum* bv genistearum should be revised.

Related to the *B. canariense* group (about 96% identity), the strains BLUT2 and ISLU207 represent two independent lineages, congruent with the ITS analysis. Nevertheless, in disagreement with this analysis, the high identity found in the *atpD* gene (near 98%) suggested that they could belong to the same species. Moreover the results of the *atpD* gene analysis are in disagreement with the identification of the strain ISLU207 as belonging to the genospecies alpha since it has less than 94% identity with respect to the strain BC-C1. These discordant results depending on the phylogenetic marker analysed showed that it is necessary to have more strains from these groups in order to establish their taxonomic status as well as that of other phylogenetic lineages also formed by single strains isolated from other *Lupinus* species (Stepkowsky *et al.,* 2007).

Although according our results and those from Stepkowsky *et al.* (2007), the strains nodulating different *Lupinus* species in Europe mostly belong to *B. canariense.* Our results showed that strains isolated on mainland Spain belong to both *B. canariense* and *B. japonicum* clusters. This finding suggests that the distribution of these two species in *Lupinus* nodules could be more related with the local ecological conditions than with its geographic (continent) location. Nevertheless, further studies on biodiversity and biogeography of strains nodulating *Lupinus* are necessary to establish more reliable conclusions.

Analysis of the *nodC* gene

The *nodC* gene determines the host range of rhizobia and it is therefore related with the host promiscuity (Roche *et al.*, 1996; Perret *et al.*, 2000; Laguerre *et al.*, 2001; Rivas *et al.*, 2007b; Iglesias *et al.*, 2007). It has been reported that restrictive hosts such as *Cicer* are nodulated by species bearing almost identical *nodC* genes (Rivas *et al.*, 2007b; Laranjo *et al.*, 2009) whereas promiscuous hosts such as *Phaseolus* or *Prosopis* are nodulated by rhizobial species carrying divergent *nodC* genes (Laguerre *et al.*, 2001; Iglesias *et al.*, 2007). Previous analyses of some isolates from nodules of legumes belonging to the *Genisteae* Tribe showed the high conservation degree of this gene (Jarabo-Lorenzo *et al.*, 2003; Kalita *et al.*, 2006) and led to the definition of the biovar genistearum within *B. canariense* and *B. japonicum* (Vinuesa *et al.*, 2005a). However, the *nodC* genes of *B. japonicum* bv genistearum strains clustered separately (identity lower than 75%) from those of *Bradyrhizobium japonicum* bv glycinearum (Figure 5). The strains from biovar genistearum nodulating *Genisteae*, including *Lupinus*, and *Ornithopus* (Tribe *Loteae*), belong to a wide phylogenetic *nodC* clade supported by a bootstrap value of 99% and with an internal similarity of about 90%. Although the rhizobia nodulating the *Genisteae* and *Ornithopus* belong to the same cross-inoculation group, the relatively low identity level of their *nodC* genes (near to 92% in some cases) indicated that these legumes are less restrictive than other hosts such as *Cicer* whose endosymbionts have almost 100% identity in their *nodC* genes (Rivas *et al.*, 2006b). Within the clade formed by the biovar genistearum strains, those nodulating *L. albus* are located in four groups (A to D) that also contain other strains isolated from *Genisteae* except in the case of group C that is formed exclusively by *L. albus* endosymbionts. The results from *nodC* analysis basically agree with those found after the analysis of the *nodA* gene since the strains from biovar genistearum are included in the *nodA* clade II including the strains BLUT1, MCLA07 and RLA08 that belong to three different subgroups of *nodA* (Stepkowski *et al.* 2007). On the basis of the *nodA* gene analysis strain BLUT1 belongs to a group that also contains isolates from other *Lupinus* species in Europe and America. However according to the results of the *nodC* gene, strains BLUT1 and MCLA07 isolated from *L. albus* in Canary Islands (geographically near to Africa) and Salamanca (mainland Spain, Europe), respectively, belong to the same group (group D) which also grouped other strains isolated from these two locations (Figure 5). The León strains (mainland Spain, Europe) and several isolates from Chile (South America) belong to a phylogenetically distant group (group A). The strains WM9 and RLA08 (Poland) isolated in Europe from *L. luteus* and *L. albus*, respectively, belong to the same group after both *nodC* and *nodA* gene analyses (Stepkowsky *et al.*, 2007). Furthermore, the *nodC* group A includes the strain BGA-1 (that was also close to the same strains on the basis of the ITS sequences) but also the strain BC-C1, a representative strain of the genospecies alpha.

The group B was constituted by miscellaneous strains isolated from different hosts and geographical origins including the strain BRE-1 representative of the genospecies beta. To this group belong the strain ISLU207, isolated in Chile (South America) and strains BLUT2 and BLUH1, isolated from *L. albus* and *L. angustifolius*, respectively, in Canary Islands. This last strain belonged to the same group as strain MCLA07 when the *nodA* gene was analysed (Stepkowski *et al.*, 2007). Therefore, some differences in the phylogenetic arrangement of *L. albus* strains BLUT1 and MCLA07 were found depending on the gene analyzed.

Nevertheless, in agreement with the results found on the basis of the *nodA* gene (Stepkowsky *et al.*, 2007), the strains from *nodC* groups B and D are dominant in Europe and Africa. Finally, it must be highlighted that the group C was exclusively formed by strains nodulating *L. albus* on different continents i.e strains BLUT3, BLUT5 and BLUT6 isolated on the Canary Islands (near to Africa), strain MCLA22 isolated on mainland Spain (Europe) and strain ISLU203 isolated in Chile (South America). Although this finding could suggest a closer coevolution among the strains from group C and their host, no significant differences in the number of nodules per plant (ranging from 20 to 30) and shoot dry weight (ranging from 300 to 400 mg/plant) were found between the strains from group C and the remaining *nodC* groups (Table 3). The presence in the groups A and C of strains isolated from Spanish and South American soils suggests that *L. albus* endosymbionts could have been dispersed from Europe to American countries together with the legume seeds. Nevertheless, as was mentioned in the case of the chromosomal genes, more strains isolated from different countries and from different cultivated and wild *Lupinus* species should be analyzed to establish the geographical distribution patterns of lupine endosymbionts.

In summary, the results of chromosomal and symbiotic markers analysis from this study showed that the *L. albus* endosymbionts isolated in different continents belong to at least four genetic lineages within the genus *Bradyrhizobium*. However, the existence of some discordant results among these markers showed that a revision of genus *Bradyrhizobium* through a polyphasic study is necessary to establish the taxonomic status of several phylogenetic groups within this genus. For this, additional strains isolated from different legumes of tribes *Genisteae* and *Loteae* should be analysed in order to describe the potential new species detected in this and previous works and to establish more reliable conclusions about the biogeography and host range of these species. The results of this work also showed a certain degree of coevolution between chromosomes and the *nodC* gene in *L. albus* isolates since the strains of *B. canariense* carry *nodC* genes phylogenetically related among them (groups C and D) and distant to those carried by the remaining strains. Also the strains from the chromosomal cluster of *B. japonicum* bv genistearum BGA-1, with the exception of strain ISLU203, carry phylogenetically related *nodC* genes (cluster A). Therefore a tripartite coevolution could be occurring among chromosomes, symbiotic elements and hosts at least in the case of *L. albus* nodulating strains.

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Legends to figures

Figure 1. Results of the mathematical analysis of RAPD patterns from strains isolated in this study. *B. japonicum* bv glycinearum LMG 6138^{T} (lane 1), *B. japonicum* bv genistearum BGA-1 (lane 2), *B. canariense* BTA-1^{T} (lane 3), *Bradyrhizobium* genosp. α BC-C1 (lane 4), *Bradyrhizobium* genosp. β BRE-1 (lane 5), RLA08 (lane 6), RLA09 (lane 7), RLA10 (lane 8), RLA11 (lane 9), MCLA07 (lane 10), MCLA12 (lane 11), MCLA22 (lane 12), MCLA23 (lane 13), BLUT1 (lane 14), BLUT2 (lane 15), BLUT3 (lane 16), BLUT5 (lane 17), BLUT6 (lane 18), BLUT8 (lane 19), ISLU203 (lane 20), ISLU207 (lane 21), ISLU213 (lane 22), ISLU220 (lane 23), ISLU227 (lane 24). MW: Molecular weight standard VI (Roche, Germany).

Figure 2. Neighbour-joining phylogenetic tree based on *rrs* gene sequences (1480 nt) showing the position of representative strains from each RAPD group. Bootstrap values calculated for 1000 replications are indicated. Bar, 0.2 nt substitution per 100 nt. Strains isolated from *L. albus* nodules are in bold. Asterisks indicate the strains identified as a previously described species of genus *Bradyrhizobium*.

Figure 3. Neighbour-joining phylogenetic tree based on 16S-23S rDNA intergenic sequences (725 nt) showing the position of representative strains from each RAPD group. Bootstrap values calculated for 1000 replications are indicated. Bar, 1 nt substitution per 100 nt. Strains isolated from *L. albus* nodules are in bold. Asterisks indicate the strains identified as a previously described species of genus *Bradyrhizobium*.

Figure 4. Neighbour-joining phylogenetic tree based on *atpD* gene (460 nt) showing the position of representative strains compared with strains isolated from other legume hosts mainly from *Lupinus* and other *Genisteae* and *Loteae* isolates. Bootstrap values calculated for 1000 replications are indicated. Bar, 5 nt substitution per 100 nt. Strains isolated from *L. albus* nodules are in bold. Asterisks indicate the strains identified as a previously described species of genus *Bradyrhizobium*.

Figure 5. Neighbour-joining phylogenetic tree based on *nodC* gene sequences (610 nt) showing the position of representative strains from each RAPD group. Bootstrap values

calculated for 1000 replications are indicated. Bar, 5 nt substitution per 100 nt. Strains isolated from *L. albus* nodules are in bold.

Table 1. Representative characteristics of reference strains and strains isolated from *Lupinus albus*.

Strain	**geographical location**	**source or nodulated hosts**	**Reference**	**RAPD pattern**	**ITS group**	***nodC* group**
B. japonicum bv glycinearum LMG 6138^T	China	*Glycine max*	Vinuesa *et al.* (2005)	A		NA
B. japonicum bv genistearum BGA-1	Canary Islands (Spain)	*Teline stenopetala*	Vinuesa *et al.* (2005)	B	III	A
B. canariense BTA-1^T	Canary Islands (Spain)	*Chamaecytisus proliferus*	Vinuesa *et al.* (2005)	C	I*	D
Bradyrhizobium genosp. α BC-C1	Canary Islands (Spain)	*Chamaecytisus proliferus*	Vinuesa *et al.* (2005a)	D	IV	A
Bradyrhizobium genosp. β BRE-1	Canary Islands (Spain)	*Teline canariense*	Jarabo-Lorenzo *et al.* (2003)	E		B
RLA08	Léon (Spain)	*L. albus*	Rivas *et al.* (2009)	F	III	A
RLA09	Léon (Spain)	*L. albus*	Rivas *et al.* (2009)	G	III	A
RLA10	Léon (Spain)	*L. albus*	Rivas *et al.* (2009)	H	III	A
RLA11	Léon (Spain)	*L. albus*	Rivas *et al.* (2009)	I	III	A
MCLA07	Salamanca (Spain)	*L. albus*	Rivas *et al.* (2009)	J	I*	D
MCLA12	Salamanca (Spain)	*L. albus*	Rivas *et al.* (2009)	K	II*	D
MCLA22	Salamanca (Spain)	*L. albus*	Rivas *et al.* (2009)	L	I*	C
MCLA23	Salamanca (Spain)	*L. albus*	Rivas *et al.* (2009)	M	II*	D
BLUT1	Canary Islands (Spain)	*L. albus*	Jarabo-Lorenzo *et al.* (2003)	N	I*	D
BLUT2	Canary Islands (Spain)	*L. albus*	Jarabo-Lorenzo *et al.* (2003)	O		B
BLUT3	Canary Islands (Spain)	*L. albus*	Jarabo-Lorenzo *et al.* (2003)	P		C
BLUT5	Canary Islands (Spain)	*L. albus*	Jarabo-Lorenzo *et al.* (2003)	Q	I*	C
BLUT6	Canary Islands (Spain)	*L. albus*	Jarabo-Lorenzo *et al.* (2003)	R	I*	C
BLUT8	Canary Islands (Spain)	*L. albus*	Jarabo-Lorenzo *et al.* (2003)	S	I*	D
ISLU203	Cautín (Chile)	*L. albus*	Jarabo-Lorenzo *et al.* (2003)	T	III	C
ISLU207	Cautín (Chile)	*L. albus*	Jarabo-Lorenzo *et al.* (2003)	U	IV	B
ISLU213	Cautín (Chile)	*L. albus*	Jarabo-Lorenzo *et al.* (2003)	V	III	A
ISLU220	Valdivia (Chile)	*L. albus*	Jarabo-Lorenzo *et al.* (2003)	W	III	A
ISLU227	Valdivia (Chile)	*L. albus*	Jarabo-Lorenzo *et al.* (2003)	X	III	A

*according Safronova *et al.* (2007).

Table 2. Sequence accession numbers of genes analysed in this study corresponding to the strains isolated from *L. albus* nodules

Strains	*rrs*	ITS	*atpD*	*nodC*
MCLA07	EF694770	EF694745	FM253158, AM168301	EF694751
MCLA12	EF694741	EF694746	FM253159	EF694752
MCLA22	EF694742	EF694747	FM253160	EF604753
MCLA23	EF694743	EF694748	FM253161	EF694754
BLUT1	EU333379	EU333383	AM168276	EU333389
BLUT2	GQ863574	GQ863558	GU338032	GQ863566
BLUT3	GQ863575	GQ863559	GU338033	GQ863567
BLUT5	GQ863568	GQ863552	GU338034	GQ863560
BLUT6	GQ863569	GQ863553	*	GQ863561
BLUT8	GQ863570	GQ863554	*	GQ863562
RLA08	EF694744	EF694749	FM253166, AM168303	EF694755
RLA09	EF694744	EU333386	FM253167[¥]	EU333391
RLA11	EU333381	EU333387	FM253169[¥]	EU333392
ISLU203	GQ863571	GQ863555	GU338035	GQ863563
ISLU207	AJ558028	GQ863557	GU338036	AJ560652
ISLU213	GQ863572	GQ863556	*	GQ863564
ISLU220	GQ863573	EF990556	*	GQ863565
ISLU227	AJ558032	EU333385	*	EU333390

* *atpD* gene sequences of strains BLUT6 and BLUT8 were not deposited in databases because they are identical to that of strain BLUT5. Also, the sequences of strains ISLU213, ISLU207 and ISLU227 were identical to that of strain ISLU203.

[¥] *atpD* gene sequences of the strains RLA09 and RLA11 were obtained by other authors in a previous work and were not included in the phylogenetic tree since they are identical to that of strain RLA08.

Table 3. Symbiotic characteristics of strains isolated from *L. albus* nodules representative of each group of *nodC* gene and geographical origin.

Strains	*nodC* subgroup	NN*	SDW† (g)	SE‡ (%)
ISLU213	A	30	0.40	66.7
RLA08	A	25	0.30	50.0
ISLU207	B	21	0.36	60.3
BLUT2	B	21	0.30	50.0
ISLU203	C	20	0.35	58.3
BLUT3	C	22	0.38	63.3
MCLA22	C	20	0.36	60.0
BLUT1	D	25	0.35	58.3
MCLA23	D	20	0.40	66.7

Not significant differences were found at P=0.05 according to Fisher's Protected LSD (Least Significant Differences)

*NN: number of nodules per plant.

†SDW: Shoot Dry Weigth per plant. SDW inoculated plants/SDW non-inoculated control plants (140 p.p.m. nitrogen as NH_4NO_3). SDW-average shoot dry weight from five replicates.

‡ SE: Symbiotic efficiency.